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PRINCIPAL INVESTIGATOR: Lingyan Jiang

CONTRACTING ORGANIZATION: The Research Foundation of SUNY
Syracuse, NY 13210

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Lingyan Jiang

Email: jiangl@upstate.edu

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The Research Foundatiion of SUNY
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This Predoctoral Traineeship Award was specially intended to support Lingyan Jiang in Functional Studies of Tumor Suppressor RASSF1A in Breast Cancer. RASSF1A is a firmly established tumor suppressor, which is silenced in a variety of cancer. Our preliminary studies demonstrate RASSF1A directly interacts with a potential oncoprotein NAP1 (nucleosome assembly protein 1) through the MT-interacting region on RASSF1A. Based on these novel findings, we hypothesized that NAP1 could play an important role in regulating RASSF1A's tumor suppressor function. By directly binding to RASSF1A, NAP1 could prevent RASSF1A from interacting with and stabilizing MTs, thus reduce RASSF1A's ability to induce cell cycle arrest and to suppress cell growth. In the first 12 months of work, I have examined NAP1-RASSF1A interaction in different phases of cell cycle. Further more, I found that purified NAP1 interferes with purified RASSF1A's ability to stabilize microtubules polymerization *in vitro*. Finally I have established NAP1 knock down cell lines to further study the effect of loss of endogenous NAP1 to RASSF1A's regulation on cell cycle and growth suppression. Together, work completed has prepared me for in-depth study of the role of NAP1-RASSF1A interaction in regulation of RASSF1A's tumor suppressing function.

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Predoctoral Traineeship Award:

Functional Studies of Tumor Suppressor RASSF1A in Breast Cancer

Lingyan Jiang
Department of Pharmacology
SUNY Upstate Medical University

First Annual Progress Report
June 16, 2008 to June 15, 2009

1. Introduction

This Predoctoral Traineeship Award was specially intended to support Lingyan Jiang in Functional Studies of Tumor Suppressor RASSF1A in Breast Cancer.

RASSF1A is a firmly established tumor suppressor, which is silenced in a variety of cancer. However, the molecular mechanisms underlying its tumor suppressing function and regulation in cancers is still unclear. Our previous studies have shown that RASSF1A associates with and stabilizes microtubules (MTs) and its interactions with microtubules are important for RASSF1A's tumor suppressor function [1, 2] . Our preliminary studies demonstrate RASSF1A directly interacts with a potential oncoprotein NAP1 (nucleosome assembly protein 1) through the MT-interacting region on RASSF1A. Based on these novel findings, we hypothesized that NAP1 could play an important role in regulating RASSF1A's tumor suppressor function. We proposed that by directly binding to RASSF1A, NAP1 could prevent RASSF1A from interacting with and stabilizing MTs, thus reduce RASSF1A's ability to induce cell cycle arrest and to suppress cell growth.

We have proposed the following three specific aims in the original proposal: (1) To determine whether NAP1-RASSF1A interactions are cell cycle dependent and define the subcellular localization of RASSF1A and NAP1 interactions. (2) To investigate the effect of NAP1 and RASSF1A interactions on microtubule dynamics. (3) To determine the effect of NAP1 on RASSF1A-mediated cell cycle regulation and growth suppression in breast cancer cells. Work completed during the first 12 months of this award period will be summarized in terms of these aims specified in the original proposal.

2. Body

2.1 To determine whether NAP1-RASSF1A interactions are cell cycle dependent and define the subcellular localization of RASSF1A and NAP1 interactions.

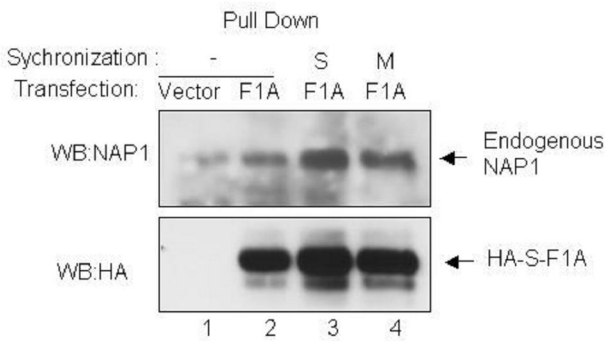

Fig. 1 NAP1 interacts with RASSF1A in both S phase and M phase. 293T cells were transfected with either vector only (lane1) or HA- and S-tagged RASSF1A expression vector (F1A, lane 2~4). Cells were then either unsynchronized (-, lane 1&2) or synchronized in S phase (S, lane 3) or M phase (M, lane 4) by 3mM Thymidine or 300ng/ul nocodazole respectively for 24h. Cell lysates were then subjected to s-tag pull down assay, and the pull down product were analyzed by WB with indicated antibodies.

To determine RASSF1A&NAP1 interaction in different cell cycle phases, we have performed pull down assay on 293T cells overexpressing S-tagged RASSF1A proteins. The interaction of NAP1 with RASSF1A was examined by Western Blot analysis of the pull down product using NAP1 specific antibodies. 293T cells were either unsynchronized or synchronized in S and M phase by thymidine block and nocodozol treatment respectively. As shown in Fig 1, the interaction of RASSF1A&NAP1 were detected in both S phase and M phase and were stronger than that seen in the unsynchronized cells.

It is well established that during M-phase of the cell cycle, microtubules rapidly assemble and disassemble to generate mitotic spindles ensuring equal divisions of sister chromatids into daughter cells. This process requires increased microtubules instability dynamics. It appears that RASSF1A, as a microtubule stabilizer, prevents the microtubules disassembly, thus put an arrest on microtubules instability dynamics and mitotic cell cycle progress [1]. NAP1 potentially prevents RASSF1A from binding to and stabilizing microtubules. So increased binding of NAP1 to RASSF1A during M phase could be one of the mechanisms the cells employ to overcome the arrest caused by RASSF1A.

The more significant enhancement of interaction in S phase came as quite a surprise to us. Our preliminary data show that RASSF1A localizes to microtubules and centrosome. Yeast NAP1 is reported as a nucleocytoplasmic shuttling protein and accumulates in the nucleus during S phase [3]. There are two possibilities to explain why RASSF1A and NAP1 interaction increased during S phase. One possibility is that NAP1 interacts with RASSF1A and translocates RASSF1A to the nucleus as it does with histones. Indeed, by fractionation, we have found that a small fraction of endogenous RASSF1A in Hela cells localizes to the nucleus. Another possibility is that a small fraction of NAP1 binds to RASSF1A on centrosome and disrupts the association of RASSF1A with tubulins on centrosome, so the centrosome could duplicate in the S phase.

To answer these questions, we plan in the future studies to 1) examine mammalian NAP1 localization in different phase of cell cycle; 2) examine where does the RASSF1A and NAP1 interaction occurs during S phase, the nucleus or centrosome? 3) examine whether NAP1 affect RASSF1A the nucleus localization and centrosome localization.

2.2 To investigate the effect of NAP1 and RASSF1A interactions on microtubule dynamics.

Our preliminary results in the original proposal showed that NAP1 directly binds to RASSF1A and their binding involves microtubule interaction domain on RASSF1A. Based on this finding we proposed that NAP1, by binding to RASSF1A, interferes with RASSF1A's ability to interact with microtubules. To test this hypothesis, we performed in vitro microtubules polymerization assay. Briefly, purified bovine tubulins (purchased from Cytoskeleton Inc.) were incubated under polymerization conditions in the presence or absence of purified RASSF1A and NAP1 proteins . We reasoned that if NAP1 could affect RASSF1A binding to microtubules, then in the presence of NAP1, RASSF1A-mediated microtubule polymerization should be lower than that seen with RASSF1A. Microtubules polymerization was monitored by increase in light absortion at 340nm. As show in Fig 2, purified RASSF1A itself enhances microtubules polymerization in a dose dependent manner. When purified NAP1 protein was added together with RASSF1A, it greatly abolished RASSF1A's ability to stimulate the polymerization reaction, the rate

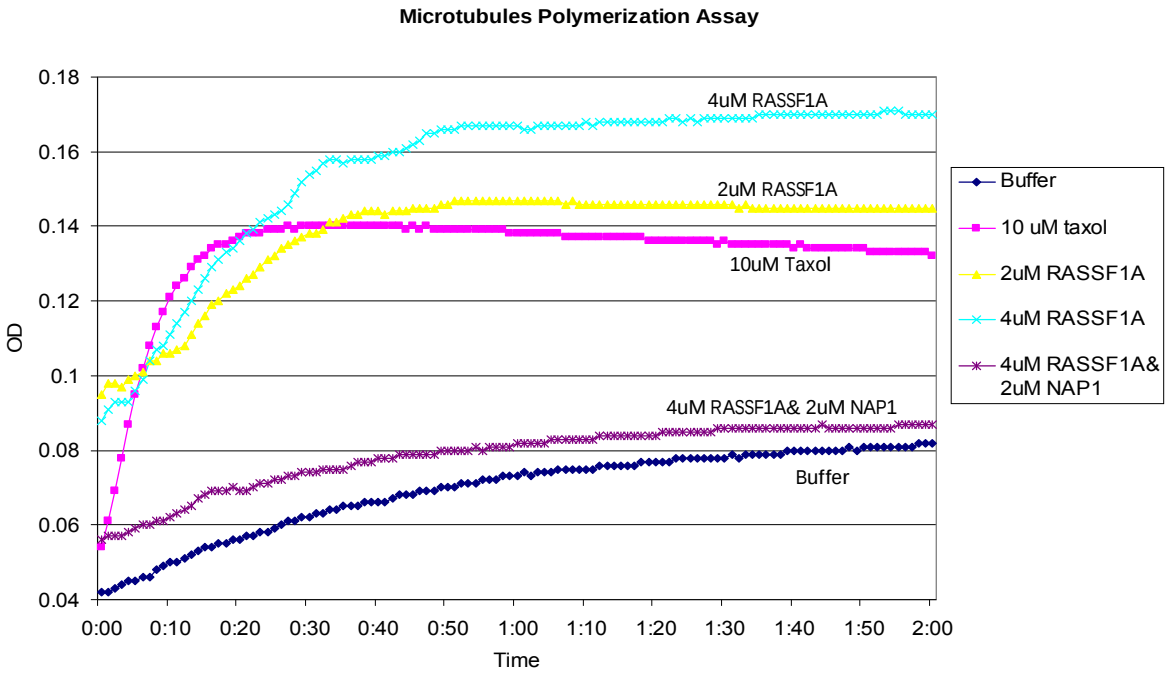

Fig. 2 Purified RASSF1A enhances microtubules polymerization, which is antagonized by purified NAP1 proteins. 800ug purified tubulin in the presence or absence of purified RASSF1A and NAP1 proteins as indicated and polymerization was measured with light absorption at 340nm at 37°C.

decreased almost to the basal level. Thus, our data suggest that NAP1 does interfere with RASSF1A's ability to stabilizes microtubules. In the future studies, we plan to examine 1) whether NAP1 also antagonizes RASSF1A's ability to stabilize polymerized microtubules inside the cells, we will determine RASSF1A's association with microtubules in the presence or absence of NAP1 by microtubules Cosedimentation assay; 2) whether NAP1's interference on RASSF1A is NAP1-RASSF1A interaction dependent. We have identified three acidic regions in NAP1 as potential RASSF1A binding region. We are currently generating NAP1 deletion mutant expressing vectors, with which we will map RASSF1A binding region on NAP1. NAP1 mutant that cannot interact with RASSF1A will be used to examine whether the effects observed are interaction dependent.

2.3 To determine the effect of NAP1 on RASSF1A-mediated cell cycle regulation and growth suppression in breast cancer cells.

Originally we proposed to co-overexpress NAP1 and RASSF1A in MCF-7, HCC38 and HCC1395 cell lines to approach this specific aim. However, in our study we found that NAP1 is expressed already at high level in most cell lines we examined. Overexpressing NAP1 on the top of the already high endogenous level might push the system to un-physiological levels. To test the hypothesis under a more physiological condition, we decided to use shRNA knock down system instead. Currently, we have established NAP shRNA in both RASSF1A expressing cell lines (MEF and Hela) and RASSF1A non-expressing cell lines (293T and MCF7). We are planning to use these cell lines to analyze

whether RASSF1A's ability to induce cell cycle arrest and growth suppression is elevated when endogenous NAP1 is depleted.

3. Key Research Accomplishments

3.1 Examined RASSF1A and NAP1 interaction inside cells in different cell cycle phases.
3.2 Determined purified NAP1 antagonizes RASSF1A's ability to stabilize microtubules polymerization in vitro.
3.3 Generated stable NAP1 knock down cell lines.
3.4 Participation in "first international RASSF symposium" and 100th AACR annual meeting.

4. Reportable Outcomes: N/A

5. Conclusion

We have examined NAP1-RASSF1A interaction in different phases of cell cycle. We will further determine the cell cycle phase and subcellular localization of NAP1-RASSF1A interaction. This piece of information will greatly facilitate us determine the mechanisms of NAP1's regulation on RASSF1A inside the cells. Further more, we found that purified NAP1 interferes with purified RASSF1A's ability to stabilize microtubules polymerization in vitro, which could occurs in the cells also. Finally we have established NAP1 knock down cell lines to study the effect of loss of endogenous NAP1 to RASSF1A's regulation on cell cycle and growth suppression. Together, work completed during the first 12 months has prepared me for in-depth study of the role of NAP1-RASSF1A interactions in RASSF1A's tumor suppressing function.

6. References

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3. Miyaji-Yamaguchi M, et al., *Involvement of nucleocytoplasmic shuttling of yeast Nap1 in mitotic progression.*. Mol Cell Biol. 2003 Sep;23(18):6672-84.

7. Appendices : N/A